TECHNICAL REPORT
NATICK/TR-03/018

AD_____

SENSITIVITY OF SPORES TO HYDROSTATIC PRESSURE: MECHANISMS OF INACTIVATION, INJURY AND REPAIR PHASE II

by
Dallas G. Hoover*
Adrienne E. H. Shearer*
Anthony Sikes
and
C.P. Dunne

*University of Delaware
Newark, DE 19717-1303

March 2003

Final Report
September 1995 - June 1999

U.S. Army Soldier and Biological Chemical Command
Soldier Systems Center
Natick, Massachusetts 01760-5018

DISCLAIMERS

The findings contained in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

Citation of trade names in this report does not constitute an official endorsement or approval of the use of such items.

DESTRUCTION NOTICE

For Classified Documents:

Follow the procedures in DoD 5200.22-M, Industrial Security Manual, Section II-19 or DoD 5200.1-R, Information Security Program Regulation, Chapter IX.

For Unclassified/Limited Distribution Documents:

Destroy by any method that prevents disclosure of contents or reconstruction of the document.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
14-03-2003	Final	September 1995 - June 1999

4. TITLE AND SUBTITLE
SENSITIVITY OF SPORES TO HYDROSTATIC PRESSURE: MECHANISMS OF INACTIVATION, INJURY AND REPAIR PHASE II

5a. CONTRACT NUMBER
DAAK60-95-C-2060

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Dallas G. Hoover*, Adrienne E. H. Shearer *, Anthony Sikes** and C.P. Dunne**

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
*Department of Animal and Food Science
035 Townsend Hall
University of Delaware
Newark, DE 19717-1303

8. PERFORMING ORGANIZATION REPORT NUMBER
NATICK/TR-03/018

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
**U.S. Army Soldier and Biological Chemical Command
Soldier Systems Center
ATTN: AMSSB-RCF-P(N)
Natick, MA 01760-5018

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Over the past fifteen years, it has been established that high-hydrostatic pressure processing (HPP) has significant potential to function as an effective nonthermal method in the destruction of microorganisms in foods with little effect on the sensory qualities of the food product. However, bacterial endospores are very pressure-resistant and have been shown to require a hurdle approach for inactivation when HPP is employed. This study investigated the inhibitory effect of approved emulsifiers (sucrose esters) in comparison to the widely studied monoglyceride, monolaurin (Lauricidin™) on *Bacillus* sp., *Clostridium sporogenes*, and *Alicyclobacillus* sp. The combined treatments of sucrose laurate, HPP and mild heat were evaluated on spores of *Bacillus* and *Alicyclobacillus* in foods. The minimum inhibitory concentrations (MICs) of the sucrose esters were higher for *Bacillus*, *Clostridium*, and *Alicyclobacillus* spp. than of Lauricidin™. Sucrose stearates and sucrose palmitate were less effective and less soluble than sucrose laurates. A combined treatment (sucrose laurate L1695 [concns 0.1 and 0.5%], 392 megaPascals [MPa] at 45°C for 10 to 15 min) provided 4.5- to 5.5-$\log_{10}$ CFU/mL reductions from initial populations of 10^6 CFU/mL for *Bacillus subtilis* 168 in milk, *Bacillus cereus* 14579 in beef, *Bacillus coagulans* 7050 in tomato juice (pH 4.5), *Alicyclobacillus* sp. N1089 in tomato juice (pH 4.5) and *Alicyclobacillus* sp. N1098 in apple juice.

15. SUBJECT TERMS
BACTERIAL SPORES, SENSITIVITY, NONTHERMAL PROCESS, MECHANISMS, INHIBITION INACTIVATION, FOOD PRESEVATION, SPORES, MICROORGANISMS, ENDOSPORES, RESISTANT BACTERIA, HYDROSTATIC PRESSURE, FOOD, SUCROSE LAURATE, EMULSIFIERS, SPORE RESISTANCE

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			A. Sikes/C.P. Dunne
U	U	U	SAR	43	19b. TELEPHONE NUMBER *(Include area code)* (508) 233-4383 or 5514

TABLE OF CONTENTS

LIST OF FIGURES

FIGURES (Cont'd)

LIST OF TABLES

PREFACE AND ACKNOWLEDGEMENTS

Dallas G.Hoover, Cynthia Stewart and Adrienne E.H. Shearer, Department of Food Science, University of Delaware, Newark, DE, conducted this study, under the supervision of Mssrs. C. Patrick Dunne, Project Manager, and Anthony Sikes, contracting officer technical representative (COTR). This study, initiated on 2 September 95 and ended 6 June 99, was divided into two phases. In Phase I of the project "Sensitivity of Spores to Hydrostatic Pressure: Mechanisms of Inactivation, Injury and Repair", the response of spores of non-pathogenic *Bacillus* and *Clostridium* spp. to high hydrostatic pressure alone, and in combination with other processing adjuncts, was evaluated.

Phase II examined the mechanism of pressure-induced inactivation and damage to spores of *Bacillus* and *Clostridium* in the presence of various processing aids, e.g., sucrose laurate.

During the course of this investigation, a novel process (invention) for treating foods was discovered. The discovery relates to the combination treatment of foodstuffs with super-atmospheric pressure, sucrose ester, and mild heat (< cooking temperatures), which has shown to be bactericidal against sporulated or sporeforming microorganisms, *Bacillus* spp.

The project was funded under Contract Award Number: DAAK60-95-C-2060.

We gratefully acknowledge Jon J. Kabara (Med-Chem Labs, Inc., Galena, IL) for supplying the Lauricidin™ and Isabel Walls and Rolenda Chuyate of the National Food Processors Association for supplying the *Alicyclobacillus* cultures.

SENSITIVITY OF SPORES TO HYDROSTATIC PRESSURE: MECHANISMS OF INACTIVATION, INJURY AND REPAIR PHASE II

INTRODUCTION

The thermal processing of foods is a broadly effective and relatively inexpensive method of preserving foods dependent upon the destruction of spoilage and pathogenic microorganisms in foods (as well as degradative enzymes natural to the food). Unfortunately, heat processing can also reduce nutrient content dramatically and modify the fresh-like organoleptic qualities of foods. In addition, heating may limit the types of packaging materials available for use to those that can withstand high processing temperatures. The use of high hydrostatic pressure processing (HPP) as a means of food preservation was first investigated in the late 1800's by Hite (1899), and has received renewed attention as a result of the heightened interest in the development of alternatives to heat processing. Pressures of 300 to 700 MPa are generally effective at reducing or eliminating vegetative bacteria (Patterson et al., 1995), but bacterial spores have demonstrated resistance to pressures of 981 MPa for 40 min and 588 MPa for 120 min (Nakayama et al., 1996). Studies have shown that the antimicrobial effect of HPP is enhanced when used in conjunction with other treatments including heat (Roberts and Hoover, 1996; Okazaki et al., 1996), acidification (Roberts and Hoover, 1996), carbon dioxide (Haas et al., 1989), and antimicrobial substances such as nisin (Roberts and Hoover, 1996), and sucrose palmitic acid ester (Hayakawa et al., 1994a). The efficacy of HPP is also improved with pressure oscillations to induce germination of spores at low pressures followed by slightly elevated pressures to destroy the remaining non-dormant cells (Hayakawa et al., 1994a; b).

Monoglycerides have been used as emulsifiers by the food industry since 1932 (Kabara, 1993). Some medium-chain length monoglycerides, particularly monolaurin, also have antimicrobial activity. Monolaurin (Lauricidin™) has antifungal (Kato, 1981; Lisker and Paster, 1982), antiviral (Hierholzer and Kabara, 1982), and antibacterial (Blasyzk and Holley, 1998; Oh and Marshall, 1992; Tsuchido et al., 1981; Wang and Johnson, 1992; Baker et al., 1982) properties, including cells adherent to surfaces (Oh and Marshall, 1995) and spores (Kimsey et al., 1981). The antimicrobial effect of monolaurin is enhanced when used in conjunction with eugenol (Blaszyk and Holley, 1998), heat (Tsuchido et al., 1981), certain acids (Oh, 1993), and pH values below 6 (Oh and Marshall, 1992). Monolaurin is generally imperceptible by most taste panelists at 500 ppm or less (Kabara, 1993); however, monolaurin has at least two limitations as an antimicrobial agent. These are its minimal effect on gram-negative bacteria and the apparent competitive interactions with food lipids, starches, and proteins and loss of antimicrobial activity in foods (Shibasaki, 1982).

Sucrose esters of fatty acids are also approved for use as emulsifiers in the United States, but less is known about their antimicrobial activity than that of monolaurin. Monk et al. (1996) demonstrated antimicrobial activity of sucrose monolaurate against *Listeria monocytogenes* and a synergistic effect with EDTA. A synergistic antimicrobial effect was also observed with sucrose palmitic acid ester and HPP on *Bacillus stearothermophilus* (Hayakawa et al., 1994a).

The practice of combining preservation technologies to compensate for limitations of each and to minimize the use of extreme levels of any one treatment is also gaining interest. The objective of this study was to evaluate the inhibitory effects of a variety of sucrose esters on bacterial spores with and without HPP and mild heat in model and food systems.

MATERIALS AND METHODS

Production of Spore Crops

Spore crops of *Bacillus subtilis* 168, *Bacillus subtilis* 6051, *Bacillus coagulans* 7050, *Bacillus stearothermophilus* 10149, *Bacillus cereus* 14579, *Clostridium sporogenes* PA3679, *Alicyclobacillus acidoterrestris* N1096, and two other *Alicyclobacillus* sp. (isolates of canned tomatoes [N1089] and apple juice concentrate [N1098]) were prepared. Spores of *Bacillus* sp. were prepared by spread-plating 24- to 48-h nutrient broth cultures onto nutrient agar (NA). *B. cereus* 14579 was plated onto Schaeffer's sporulation agar (Harwood and Cutting, 1990). Plates were incubated for approximately one week at 37 °C or at 55 °C for *B. stearothermophilus* 10149 and 32 °C for *B. cereus* 14579. Sporulation was assessed by phase-contrast microscopy. Spores were harvested when predominant (>90%).

Spores of *C. sporogenes* PA3679 were prepared by spread-plating 24- to 48-h reinforced clostridial medium (RCM) cultures onto reinforced clostridial agar (RCA). RCA plates were incubated anaerobically at 37 °C for approximately one week until spores predominated (>90%) as determined by phase-contrast microscopy. Multiple attempts to sporulate *C. perfringens* 13124 were made in various recommended sporulation media including Duncan-Strong broth (M45) and sporulation broth for *C. perfringens* (M140) at 30 °C and 37 °C with and without pH adjustment (FDA, 1998). Throughout the incubation period, rods were replaced by smaller spherical structures, but refractivity was not prominent and no cells were recovered after a heat shock of 75 °C for 20 min.

Alicyclobacillus sp. was grown at 42 °C for 5 to 7 d in K medium (per liter, 2.5 g yeast extract, 5.0 g peptone, 1.0 g dextrose, 1.0 mL Tween 80, pH-adjusted to 3.9 with 1M malic acid). Spread plates of potato dextrose agar (PDA) acidified to pH 3.6 with malic acid were inoculated with growth of *Alicyclobacillus* sp. in K medium. PDA plates were incubated aerobically at 42 °C for 10 to 19 d until spores predominated as determined by phase-contrast microscopy.

Spores of *Bacillus*, *Clostridium*, and *Alicyclobacillus* were harvested from NA, RCA, and PDA, respectively, with sterile distilled water and a bent-glass rod. Spore crops of *Bacillus* and *Clostridium* were washed according to the method of Nicholson and Setlow (1990). Spores were cleaned by alternate centrifugation and washing of the pellets with 10 mL of 1 M KCl, 0.5 M NaCl, 50 mM Tris.Cl (pH 7.2) containing 50 mg/mL lysozyme (incubated 1 h at 37 °C), 1 M NaCl, sterile distilled water, 0.05% sodium dodecyl sulfate (SDS), TEP buffer (50 mM Tris.Cl buffer, pH 7.2, with 10 mM EDTA and 2 mM phenylmethylsulfonyl fluoride), and three washes of sterile distilled water. Spores of *Alicyclobacillus* were cleaned by alternate centrifugation and washing of the pellets by sterile distilled water three times. Spore crops were stored in sterile water at 4 °C. The spore crops were evaluated by phase-contrast microscopy and enumerated by pour-plating with NA, RCA, and K agar (KA) for *Bacillus*, *Clostridium*, and *Alicyclobacillus*, respectively.

Exposure of Spores to Sucrose Esters

Spores of *B. subtilis* 168, *B. subtilis* 6051, *B. coagulans* 7050, *B. cereus* 14579, *B. stearothermophilus* 10149, *C. sporogenes* PA3679, *A. acidoterrestris* N1096, *Alicyclobacillus* sp. N1098 and N1089 (10^6 CFU/mL) were pour-plated in duplicate in NA (for *Bacillus*), KA (for *Alicyclobacillus*), or RCA (for *C. sporogenes*) amended with various concentrations of sucrose

Alicyclobacillus), or RCA (for *C. sporogenes*) amended with various concentrations of sucrose esters. The sucrose esters (Ryoto Sugar Ester, Mitsubishi Chemical Corp., White Plains, NY) evaluated for inhibitory effect were four types of sucrose stearate (S570, S970, S1570, and S1670), sucrose palmitate (P1670), and two types of sucrose laurate (L1695 and LWA1570). The antimicrobial activity of the monoglyceride, Lauricidin™ (Med-Chem Labs, Inc., Galena, IL), was compared to the sucrose esters. The sucrose stearates varied by their monoester content and hydrophilic to lipophilic balance (HLB). The sucrose laurates also differed by monoester content, HLB, and physical form. L1695 was used in powder form; LWA1570 was a viscous liquid of 40% sucrose laurate, 4% ethanol, and 56% water. All other sucrose esters were solid powders. Lauricidin™ was a pellet form of monolaurin.

Treatment by Pressure, Inhibitors, and Mild Heat

The most effective of the sucrose esters (determined during screening studies with *Bacillus* and *Alicyclobacillus*) were evaluated for synergistic effects with hydrostatic pressure and mild heat against selected sporeformers in foods with which they are commonly associated. *B. subtilis* 168, *B. cereus* 14579, and *B. coagulans* 7050 were treated in milk, beef gravy, and tomato juice, respectively. *Alicyclobacillus* N1089 and N1098 were treated in tomato juice and apple juice, respectively.

***Bacillus subtilis* 168 in Milk**

UHT milk (2% milk fat, Parmalat, Teaneck, NJ) was inoculated with *B. subtilis* 168 (10^6 CFU/mL), and sampled for an initial spore count. Sucrose laurates (LWA1570 and L1695) and

Ten mL of the milk was transferred to a polyester/polyethylene pouch, and the pouches were heat-sealed (both by Kapak Corp., Minneapolis, MN). The milk was pressurized at 392 MPa at 25 or 45 °C for 10 or 15 min (Autoclave Engineers Isostatic Press Model IP2-22-60, Erie, PA). Come-up time was approximately 2.75 min and depressurization time was approximately 40 sec. Neither come-up nor depressurization times were included in the total 10- or 15-min treatment times. Milk samples treated at 45 °C were immediately cooled in water to ambient temperature after treatment. The pouches were washed and opened aseptically. Samples were pour-plated in NA amended with the same type and concentration of sucrose ester or monolaurin as was added to the milk. The plating diluent was 0.1%-peptone water. Plates were incubated at 37 °C for a minimum of 5 d and counted manually. Negative controls were plated to assure commercial sterility of the milk. Positive controls of the inoculated milk were plated prior to addition of the chemical inhibitor, and pour-plated in unamended NA to determine initial spore counts. All experiments were replicated at least once.

The effects of individual components of the treatments (pressure [P], chemical inhibitors [SL], and heat [H]) were evaluated in addition to the combination of all three. The initial counts and pressure/heat/inhibitor experiments were conducted as previously described. Also, a product sample was drawn prior to addition of the inhibitor and pressurized at 392 MPa at 45 °C, 50 °C, or 55 °C for 10 or 15 min. This sample (P/T) was plated in agar without the inhibitor. Two other samples were collected after inhibitor addition. One of these was treated without heat (SL, for sucrose laurate L1695) and plated in agar containing the sucrose ester.

The other sample was treated at the same temperature as the pressurized samples (SL/T) and plated in agar containing the inhibitor. The exposure time for the latter was 13.5 min to match the amount of heat exposure of the pressed samples inclusive of come-up and depressurization times.

B. subtilis 168 in milk was treated with 0.1 and 0.5% L1695 and 392 MPa at 45 °C for 10 min as both individual treatments and as a combination treatment as described previously. The samples, P/SL/T, P/T, and SL/T, were plated with both NA and NA amended with sucrose laurate L1695 at 0.1 or 0.5%, to determine the need for continued exposure to sucrose laurate after the pressure treatment.

Bacillus cereus 14579 in Beef

Beef gravy baby food (Gerber Products Company, Fremont, MI, containing beef, water, corn starch, lemon juice concentrate) was inoculated with *B. cereus* 14579 (10^6 CFU/mL) and treated for 10 min at 392 MPa and 45 °C with incorporation of sucrose laurate L1695 at 0.01% as previously described. Outgrowth of *B. cereus* survivors was enumerated on NA amended with 0.01% L1695; plates were incubated at 30 °C for approximately 5 d.

Bacillus coagulans 7050 in Tomato Juice

Tomato juice (Campbell Soup Company, Camden, NJ, containing tomato juice from concentrate, salt and vitamin C) was adjusted to pH 4.5 with 1 N NaOH from an initial pH of 4.2. The juice was inoculated with *B. coagulans* 7050 (10^6 CFU/mL) and treated for 10 min at 392 MPa and 45 °C along with sucrose laurate L1695 at 1% as previously described.

Outgrowth of *B. coagulans* survivors were enumerated on tomato juice agar (TJA) amended with 1% L1695 and pH adjusted to 4.5 with filter-sterilized 1 M citric acid (Fisher, Fair Lawn, NJ). Plates were incubated at 30 °C for approximately one week. In an effort to determine the appropriate concentration of L1695, the effect of treatment with 0.1 and 1.0% in conjunction with HPP at 45 °C was determined.

Alicyclobacillus sp. N1089 in Tomato Juice

Tomato juice was also inoculated with *Alicyclobacillus* sp. N1089 (10^6 CFU/mL) and treated at 392 MPa and 45 °C for 10 min with 0.01 and 0.005% L1695 as previously described. Outgrowth of surviving *Alicyclobacillus* spores was enumerated on TJA (pH 4.5). Plates were incubated at 42 °C for approximately 3 weeks.

Alicyclobacillus sp. N1098 in Apple Juice

Apple juice from concentrate (America's Choice) was inoculated with *Alicyclobacillus* sp. N1098 (10^6 CFU/mL) and treated at 392 MPa and 45 °C for 10 min with 0.025, 0.04, and 0.045% L1695. Outgrowth of *Alicyclobacillus* survivors were enumerated on KA amended with the same concentrations of L1695 as the juice and the pH adjusted to approximately 3.8 with filter-sterilized 1 M malic acid (Acros Organics, NJ). The plates were incubated approximately 3 weeks at 42 °C.

RESULTS AND DISCUSSION

Inhibition by Sucrose Esters and Lauricidin™

With the exception of *B. stearothermophilus* 10149 at 0.5% S1670, outgrowth of all *Bacillus* sp. (data not shown) and *Alicyclobacillus* sp. (Table 1) was not affected by the presence of sucrose stearates at levels of 0.5%. *C. sporogenes* PA3679 was nearly completely inhibited by 0.5% sucrose stearates except for S570 (Table 2). The sucrose stearates were insoluble in the agar until heated and precipitated during tempering of the agar to 45°C. *B. stearothermophilus* 10149 was generally more susceptible to the sucrose esters than the other bacilli. *Bacillus* sp., *Alicyclobacillus* sp., and *C. sporogenes* PA3679 were inhibited by 0.5, 0.1, and 0.5% LWA1570, respectively. Sucrose laurate L1695 was more effective on *Alicyclobacillus* sp. and *C. sporogenes* PA3679 at 0.05%, but the concentration needed to inhibit all *Bacillus* sp. tested was >1%. The greater inhibitory effect of sucrose laurates (L1695 and LWA1570) over other sucrose esters was consistent with the greater inhibitory effect of monolaurin (same chain length of fatty acid) over other monoglycerides although activity is also a function of degree of saturation (Kimsey et al., 1981; Kabara, 1993). The MIC for Lauricidin™ against all sporeformers was less than the sucrose laurates; however, Lauricidin™ was more difficult to get into solution at room temperature.

Table 1. Outgrowth of *Alicyclobacillus* spores (N1089, N1096, and N098) in the presence of sucrose esters and Lauricidin™.

Inhibitor Conc. (%)	Sucrose Stearate				Sucrose Palmitate	Sucrose Laurate		Lauricidin™
	S570	S970	S1570	S1670	P1670	L1695	LWA 1570	
0.001	NT	NT	NT	NT	NT	NT	NT	+
0.005	NT	NT	NT	NT	NT	+	NT	+
0.01	NT	NT	NT	NT	NT	+	+	-
0.05	NT	NT	NT	NT	NT	-	+(<10 CFU/mL)	-
0.1	NT	NT	NT	NT	+	-	-	-
0.5	+	+	+	+	+ -(N1089)	NT	-	NT
1.0	NT	NT	NT	NT	NT	-	NT	NT

NT Not tested.
\+ Positive for growth of all three *Alicyclobacillus* sp. tested.
\- No growth of three *Alicyclobacillus* sp. tested.

Table 2. Outgrowth of *Clostridium sporogenes* PA3679 spores in the presence of sucrose esters and Lauricidin™.

Inhibitor Conc. (%)	Sucrose Stearate				Sucrose Palmitate	Sucrose Laurate		Lauricidin™
	S570	S970	S1570	S1670	P1670	L1695	LWA 1570	
0.001	NT	NT	NT	NT	NT	NT	NT	+
0.005	NT	NT	NT	NT	NT	NT	NT	+
0.01	NT	NT	NT	NT	NT	+	+	-
0.05	NT	NT	NT	NT	NT	-	+	-
0.1	NT	NT	NT	NT	-	-	+	-
0.5	+	+	-	-	-	NT	-	NT
1.0	NT	NT	NT	NT	NT	-	NT	NT

NT Not tested.
\+ Positive for growth.
\- No growth.

Effect of HPP, Inhibitors, and Mild Heat on Sporeformers in Foods

***Bacillus subtilis* 168 in milk**

The most effective of the sucrose esters, the sucrose laurates, and Lauricidin™ were evaluated for effectiveness when used in conjunction with pressure and mild heat. Since LWA1570 and Lauricidin™ were effective alone at 0.5 and 0.01%, respectively, against *Bacillus* sp. (Table 3), these two inhibitors were tested at ambient temperature with HPP at 392 MPa. With Lauricidin™ (0.001%) and 392 MPa pressure applied for 10 min, there was no reduction in spore counts at 25 °C while at 45 °C, a 3-$\log_{10}$ reduction was observed for *B. subtilis* 168 from an initial population of 10^6 CFU/mL (Table 4). When the Lauricidin concentration was increased to 0.0025%, the inhibitory effect was the same at 25 and 45°C with or without pressure (Table 4). Because of the low concentration and small volumes of milk tested, Lauricidin was added by serial dilution where appropriate. Prior dissolution of Lauricidin with mild heat would probably be necessary regardless, because of the uncertain solubility in milk at room temperature.

LWA1570 (0.05 and 0.1%) and 10 min of 392 MPa pressure provided reductions of approximately 4- to 5-$\log_{10}$ CFU/mL, respectively, for *B. subtilis* 168 in milk at ambient temperature; however, the plate counts were erratic in that 10-fold reductions were not apparent in the dilution series and the plates were countable only when the milk was diluted adequately (10^{-2} dilution). This would suggest that the apparent effectiveness of LWA1570 might have been less had the spores been incubated in the milk rather than plated in agar. The effectiveness of LWA1570 is worthy of further investigation, but the recovery methods must be modified to increase confidence in the results.

Table 3. Outgrowth of *Bacillus* spores (*B. subtilis* 168, *B. subtilis* 6051, *B. coagulans* 7050, *B. cereus* 14579 and *B. stearothermophilus* 10149) in the presence of sucrose esters and Lauricidin™.

Inhibitor Conc. (%)	Sucrose Palmitate	Sucrose Laurate		Lauricidin™
	P1670	L-1695	LWA-1570	
0.001	NT	NT	NT	-
0.005	NT	NT	NT	- (<10 CFU/mL) - (168, 6051, 10149)
0.01	NT	NT	-	-
0.05	NT	-(only 4579 tested)	-	-
0.1	-	- - (14579)	- (< 10 CFU/mL) - (14579)	-
0.5	- - (10149)	- - (10149)	-	-
1.0	NT	- - (10149)	NT	NT

NT Not tested

\+ Positive for growth for all species of *Bacillus* tested.

\- Negative for growth for all species of *Bacillus* tested and where indicated in parentheses as exception.

Table 4. Effect of 392 MPa, inhibitors, and 45°C on *Bacillus* sp. in foods.

Food	Microbe	Process Conditions				Initial Population (CFU/mL)	Final Population (CFU/mL)
		Pressure (MPa)	Temp (C)	Inhibitor (%)	Time (min)		
Milk	*B. subtilis* 168	392	45	L1695 (0.1)	10	3.4×10^6	2.2×10^3
			45	L1695 (0.5)	10	2.6×10^6	7.8×10^2
			45	L1695 (1.0)	10	3.9×10^6	4.6×10^2
			45	L1695 (1.0)	15	2.6×10^6	6.0×10^1
			45	Lauricidin™ (0.001)	10	4.0×10^6	5.3×10^3
			45	Lauricidin™ (0.001)	15	2.6×10^6	1.5×10^3
			25	Lauricidin™ (0.001)	10	3.5×10^6	1.6×10^6
			25	Lauricidin™ (0.0025)[a]	10	2.6×10^6	5.4×10^2
			45	Lauricidin™ (0.0025)[a]	10	2.6×10^6	4.7×10^2
			25	LWA1570 (0.05)[b]	10	3.8×10^6	6.1×10^2
			25	LWA1570 (0.1)[b]	10	3.5×10^6	5.7×10^1
TJ	*B. coagulans* 7050	392	45	L1695 (0.1)	10	1.5×10^7	4.8×10^3
			45	L1695 (1.0)	10	1.5×10^7	3.7×10^1

[a] Effect was the same without pressure.

[b] Plate counts erratic.

Higher concentrations of L1695 were needed to achieve levels of reduction similar to Lauricidin™ for spore counts of *B. subtilis* 168 in milk. Solubility of L1695 was greater than the solubility observed for Lauricidin ™. Results obtained for L1695 were more reliable using plate counts than those observed with LWA1570. Reduced temperatures were not tested with L1695 as the need for mild heat with HPP was demonstrated in Phase I. Concentrations of 0.1, 0.5, and 1% L1695 and 392 MPa applied for 10 min at 45 °C provided 3-, 3.5-, and 4-$\log_{10}$ reductions, respectively, (Table 4) from an initial inoculum of 10^6 CFU/mL of *B. subtilis*. Increasing the pressurization time to 15 min at 45 °C with 1% L1695 provided a 5-$\log_{10}$ reduction (Table 4). Increasing the temperature to 50 and 55 °C for 15 min at 392 MPa with 1% L1695 provided no additional reduction of *B. subtilis* 168 spores than treatment at 45 °C under the same conditions (Fig. 3 and 1). The greatest reduction of *B. subtilis* 168 in milk found in this study was approximately 4.5-$\log_{10}$ CFU/mL with 1.0% L1695 and a 15-min HPP treatment at 45°C.

To quantify the inactivation of spores versus the inhibition of outgrowth, *B. subtilis* 168 was treated in milk with 0.1 and 0.5% L1695, 392 MPa at 45°C for 10 min and plated with and without sucrose laurate in the media (Fig. 2 and 4). The effect of pressure and mild heat gave a reduction of approximately 1.5-$\log_{10}$. With the addition of L1695 (0.1%) to the milk and the plating medium, the inactivation was improved to approximately 3 $\log_{10}$ CFU/mL after pressure treatment. When pressure-treated in the presence of sucrose laurate, but plated without the ester in the agar, spore counts were slightly less than that provided by pressure and mild heat without the ester. Results were the same whether the sucrose laurate was added before or after pressurization (Fig. 1, P/SL/T in NA/SL vs. P/T in NA/SL).

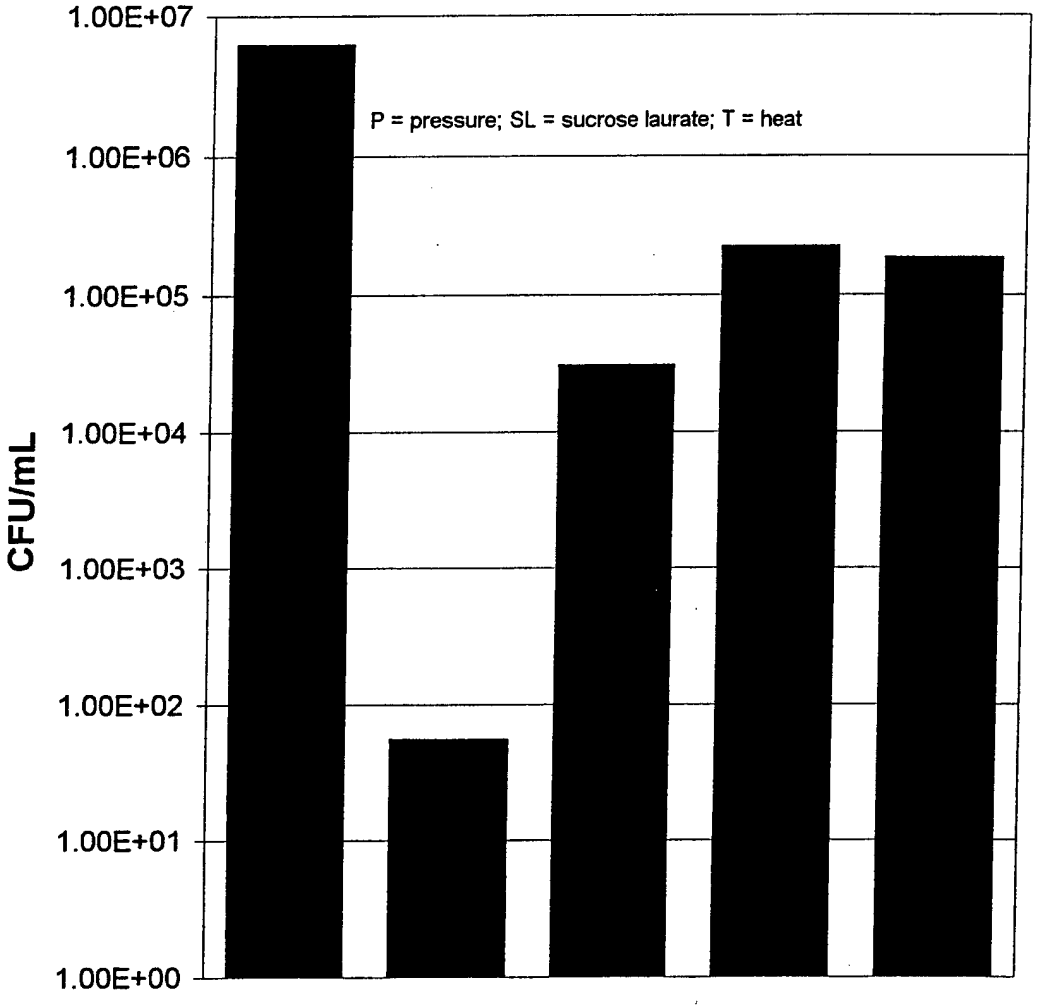

Fig. 1. Inhibition of *Bacillus subtilis* 168 in milk by individual and combined treatments of pressure (392 MPa) for 15 min at 50 °C and sucrose laurate [1% (w/v) L1695], and plated on nutrient agar.

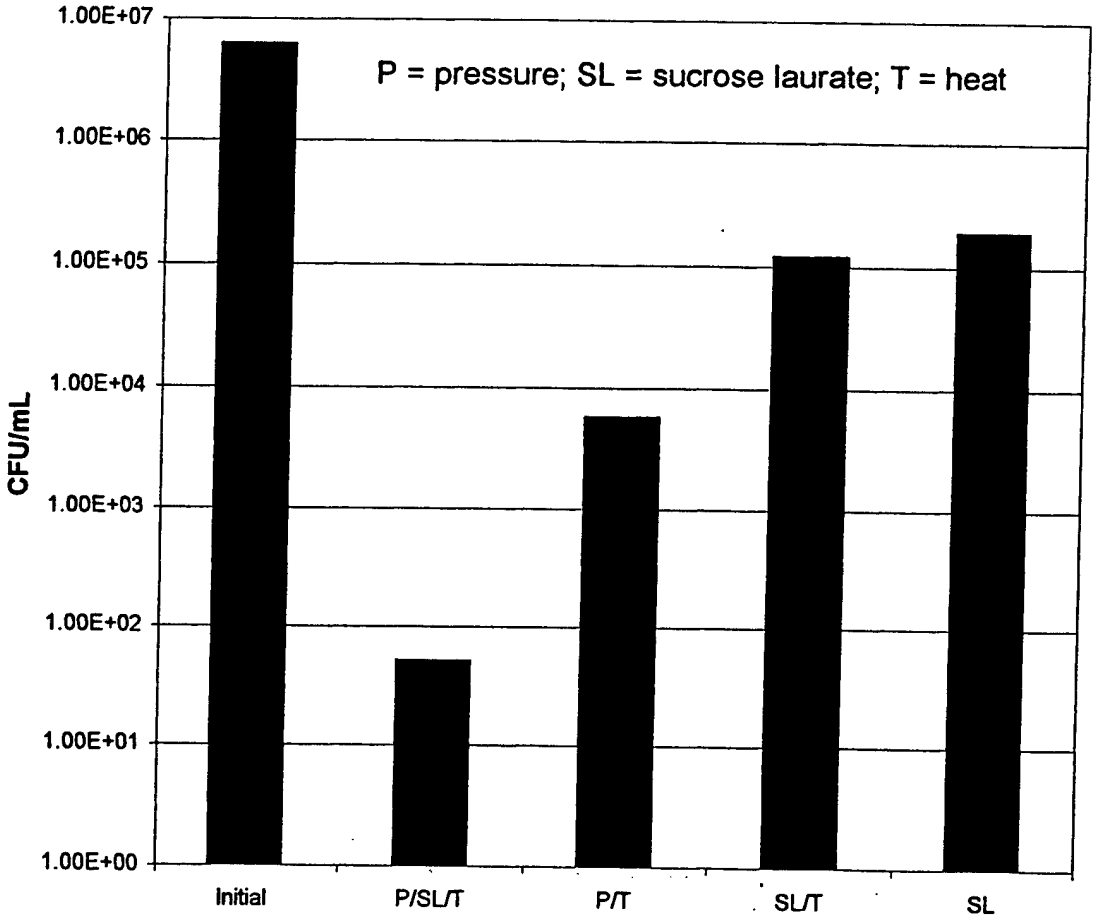

Fig. 2. Inhibition of *Bacillus subtilis* 168 in milk by individual and combined treatments of pressure (392 MPa) for 15 min at 55 °C and sucrose laurate [1% (w/v) L1695], and plated on nutrient agar.

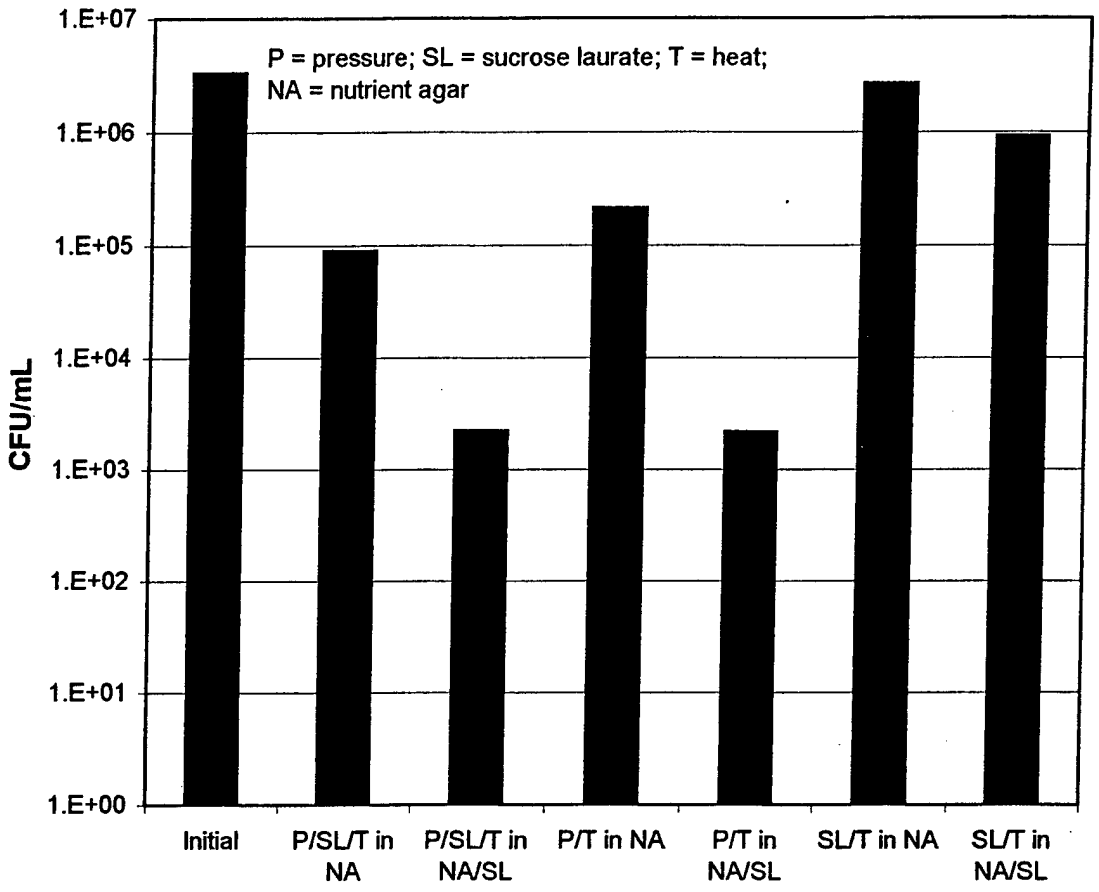

Fig. 3. Inhibition of *Bacillus subtilis* 168 in milk by individual and combined treatments of pressure (392 MPa) for 10 min at 45 °C and sucrose laurate [0.1% (w/v) L1695], and plated on nutrient agar with and without sucrose laurate

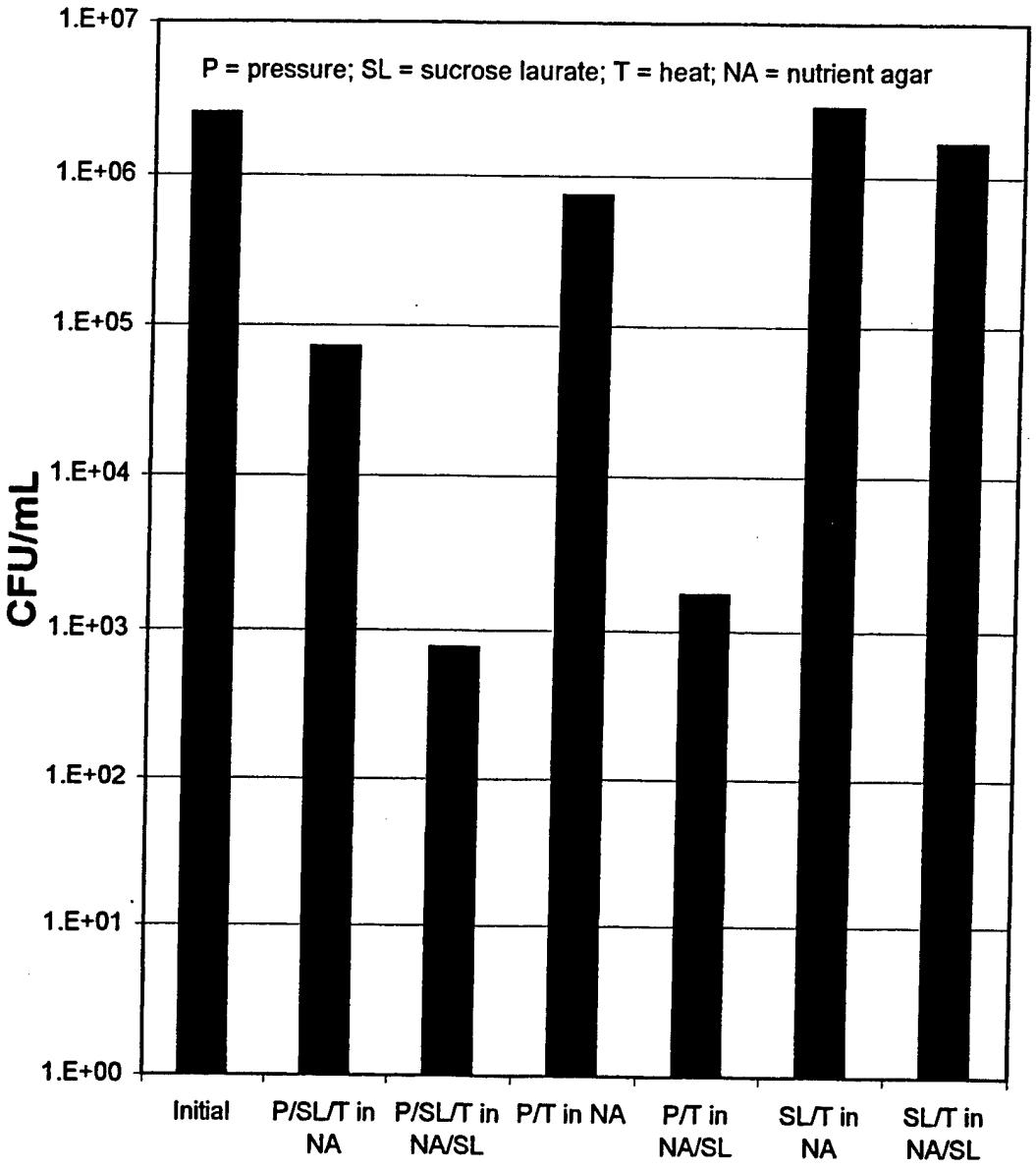

Fig. 4. Inhibition of *Bacillus subtilis* 168 in milk by individual and combined treatments of pressure (392 MPa) for 10 min at 45 °C and sucrose laurate [0.5% (w/v) L1695], and plated on nutrient agar with and without sucrose laurate

Bacillus cereus 14579 in Beef

B. cereus 14579 was less resistant to sucrose laurates L1695 and LWA1570 than *B. subtilis* 168 and 6051 for the screening studies in agar (Table 1). Similarly, the concentration of L1695 needed to achieve a reduction of approximately 5-$\log_{10}$ for *B. cereus* 14579 in beef was only 0.01% when combined with pressure at 45 °C for 10 min (Fig. 5), as compared to 1.0% L1695 required for *B. subtilis* in milk. The contribution of pressure to the inhibition and inactivation of *B. cereus* was greater than that provided by sucrose laurate alone or sucrose laurate with mild heat, neither of which had an effect on spore counts. However, when sucrose laurate was used in conjunction with pressure and mild heat, an additional 1-$\log_{10}$ reduction was observed as compared to use of pressure and mild heat alone.

Bacillus coagulans 7050 in Tomato Juice (pH 4.5)

Sucrose laurate L1695 at 1% with 10 min at 392 MPa and 45 °C reduced a 10^6 CFU/mL initial inocula of *B. coagulans* 7050 by nearly 5 $\log_{10}$ (Fig. 6). In contrast to observations with *B. cereus* 14579, the contributions of sucrose laurate alone and with the addition of heat on *B. coagulans* were greater (approximately 2 $\log_{10}$) than treatments employing just pressure and mild heat. *B. coagulans* and *B. subtilis* demonstrated similar susceptibilities to sucrose laurate L1695 when screened in agar for MICs; therefore, the additional 1-$\log_{10}$ reductions observed with the same treatment in tomato juice versus milk were possibly due to the reduction in pH or other inhibitory constituents of the tomato juice.

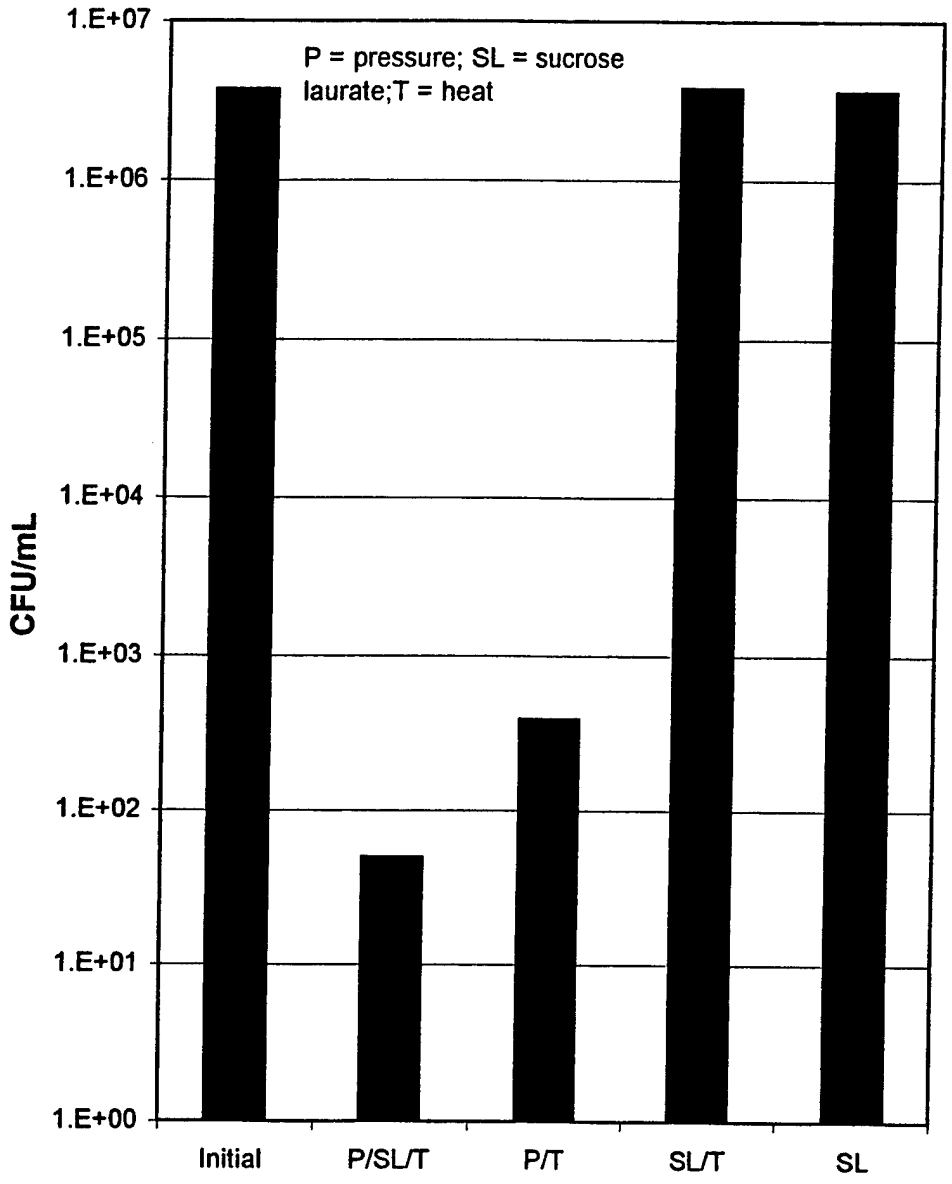

Fig. 5. Inhibition of B*acillus cereus* 14579 in beef by individual and combined treatments of pressure (392 MPa) for 10 min at 45 °C and 0.01% sucrose laurate, and plated on nutrient agar

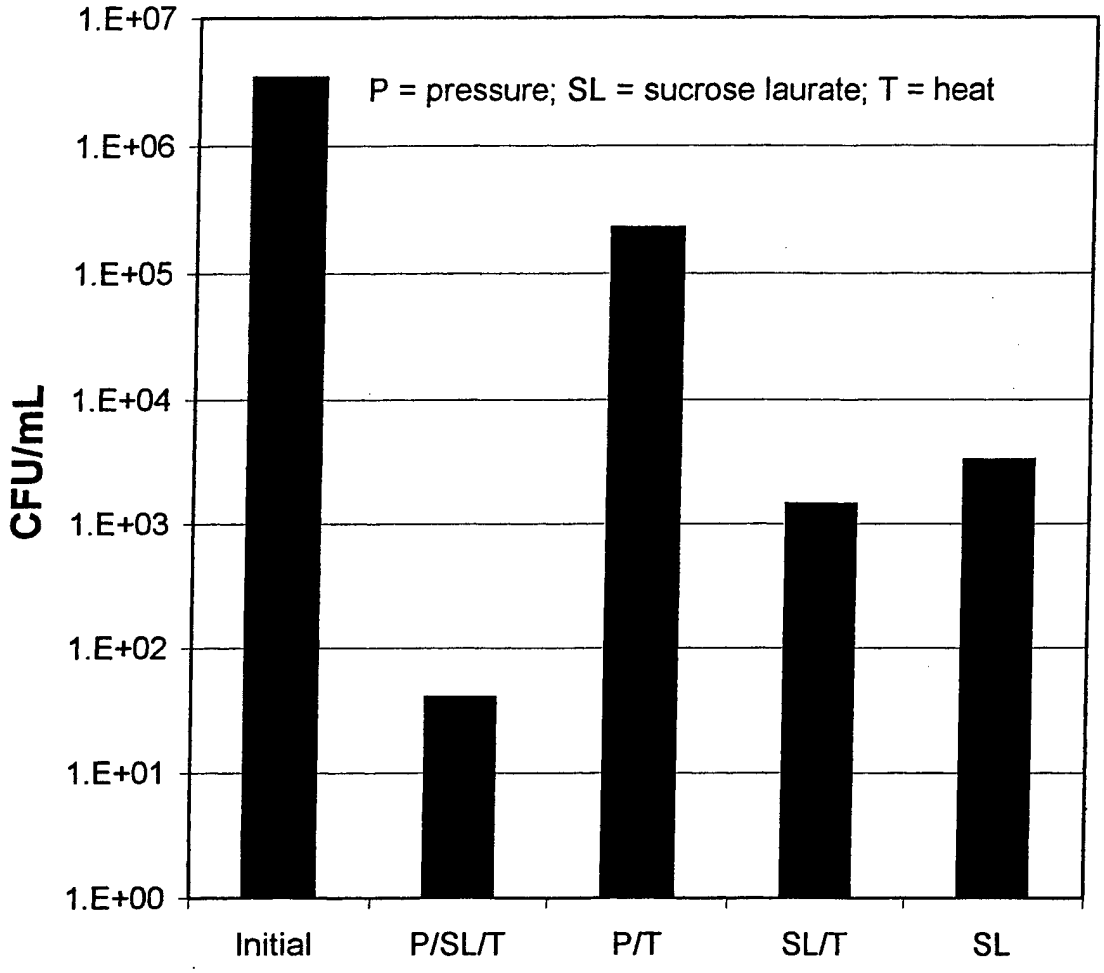

Fig. 6. Inhibition of *Bacillus coagulans* 7050 in tomato juice by individual and combined treatments of pressure (392 MPa) for 10 min at 45 °C and sucrose laurate [1% (w/v) L1695], and plated on nutrient agar

***Alicyclobacillus* N1089 in tomato juice (pH 4.5)**

A 3-$\log_{10}$ reduction of *Alicyclobacillus* N1089 was observed with the combined treatment of 0.005% L1695 and 10 min at 392 MPa and 45 °C (Fig. 7). At this concentration of L1695, the effects of pressure/mild heat and sucrose laurate/mild heat on spore count reduction was nearly equal, and the combined effect was cumulative for the two treatments applied separately. When the concentration of L1695 was increased to 0.01%, no outgrowth of spores was observed with the combined treatment of pressure/sucrose laurate/mild heat; the effect was the same with L1695 at 0.01% without pressure (Fig. 8). Interestingly, the effect of 0.01% L1695 was greater in tomato juice and tomato juice agar (pH 4.5) than when MICs were determined in KA (pH 3.8). In KA, spore outgrowth was observed at 0.01% L1695. Of the two sporeformers of concern for outgrowth in tomato products at pH 4.5, *Alicyclobacillus* N1089 should be easily inhibited by the treatments necessary for inhibition of *B. coagulans* 7050.

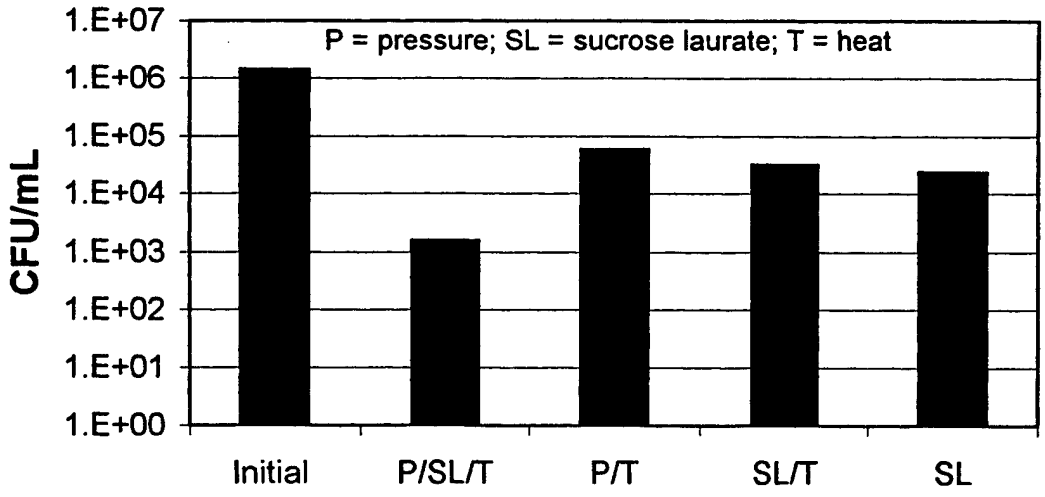

Fig. 7. Inhibition of *Alicyclobacillus* N1089 in tomato juice by individual and combined treatments of pressure (392 MPa) for 10 min at 45 °C and sucrose laurate [0.005% (w/v) L1695], and plated on nutrient agar.

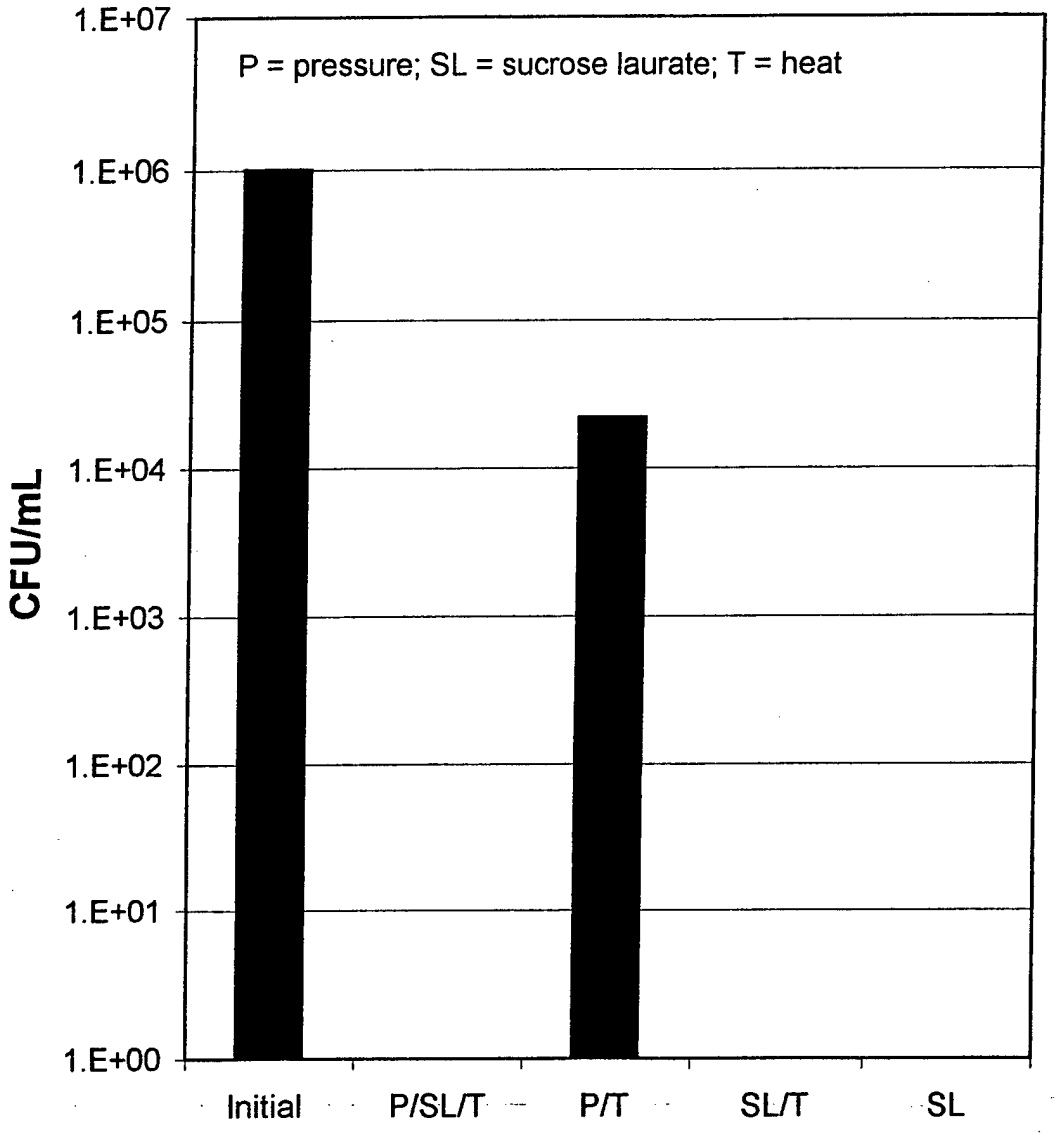

Fig. 8. Inhibition of *Alicyclobacillus* N1089 in tomato juice by individual and combined treatments of pressure (392 MPa) for 10 min at 45 °C and sucrose laurate [0.01% (w/v) L1695], and plated on nutrient agar

Alicyclobacillus N1098 in apple juice

Sucrose laurate L1695 at 0.025% in apple juice with or without heat treatment at 45°C had no inhibitory effect on *Alicyclobacillus* N1098 (Fig. 9). Treatments of 392 MPa for 10 min and 45 °C showed reductions of approximately 2-$\log_{10}$ CFU/mL with no additional reductions with the addition of 0.025% L1695. L1695 at 0.04% with pressure and mild heat treatment reduced *Alicyclobacillus* N1098 spore counts in apple juice by just 2.5 $\log_{10}$ (Fig. 10). A slight increase in the L1695 concentration to 0.045% with pressure and mild heat gave 5.5-$\log_{10}$ reductions of *Alicyclobacillus* sp. N1098 (Figure 11). *Alicyclobacillus* N1098 was completely inhibited by 0.05% L1695 when plated directly in KA for MIC determination (Table 2). If *Alicyclobacillus* is similarly effected by 0.05% L1695 in apple juice, complete inactivation should be attained with 0.005%. For practical use, it would seem most appropriate to use a slightly higher level of sucrose laurate to achieve the same effects without the use of pressure or heat, provided there was no effect on product quality and the levels were sufficient to inhibit other spoilage microorganisms (particularly fungi).

Although no formal sensory study was conducted, the concentrations of sucrose laurates and Lauricidin™ evaluated in the foods were low enough that no change in the odor, color or clarity of the foods could be detected. The most notable change in the products was temporary foaming during mixing of the sucrose ester in the foods. This could be minimized with gentle agitation or addition of previously dissolved ester.

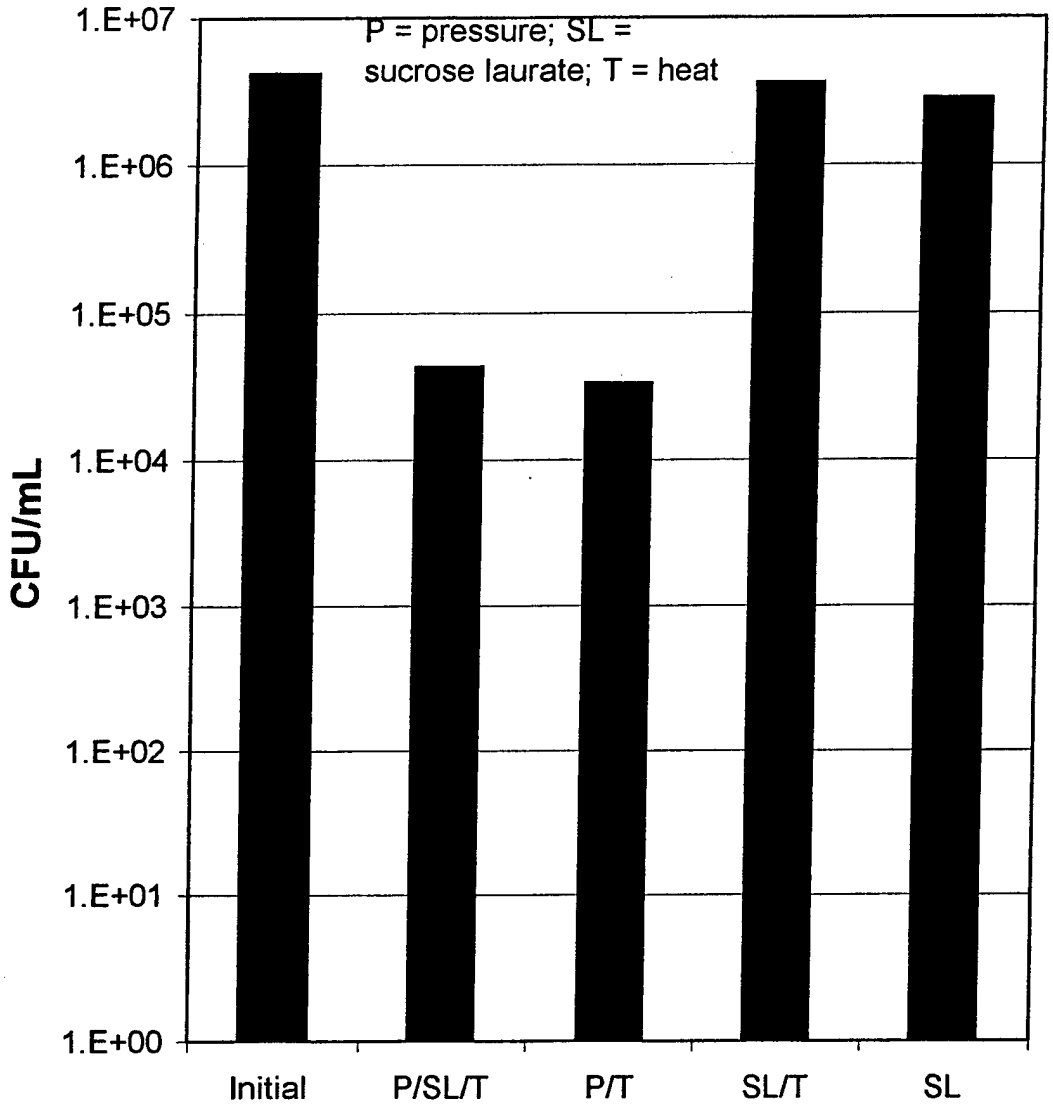

Fig. 9. Inhibition of *Alicyclobacillus* N1089 in apple juice by individual and combined treatments of pressure (392 MPa) for 10 min at 45 °C and sucrose laurate [0.025% (w/v) L1695], and plated on nutrient agar.

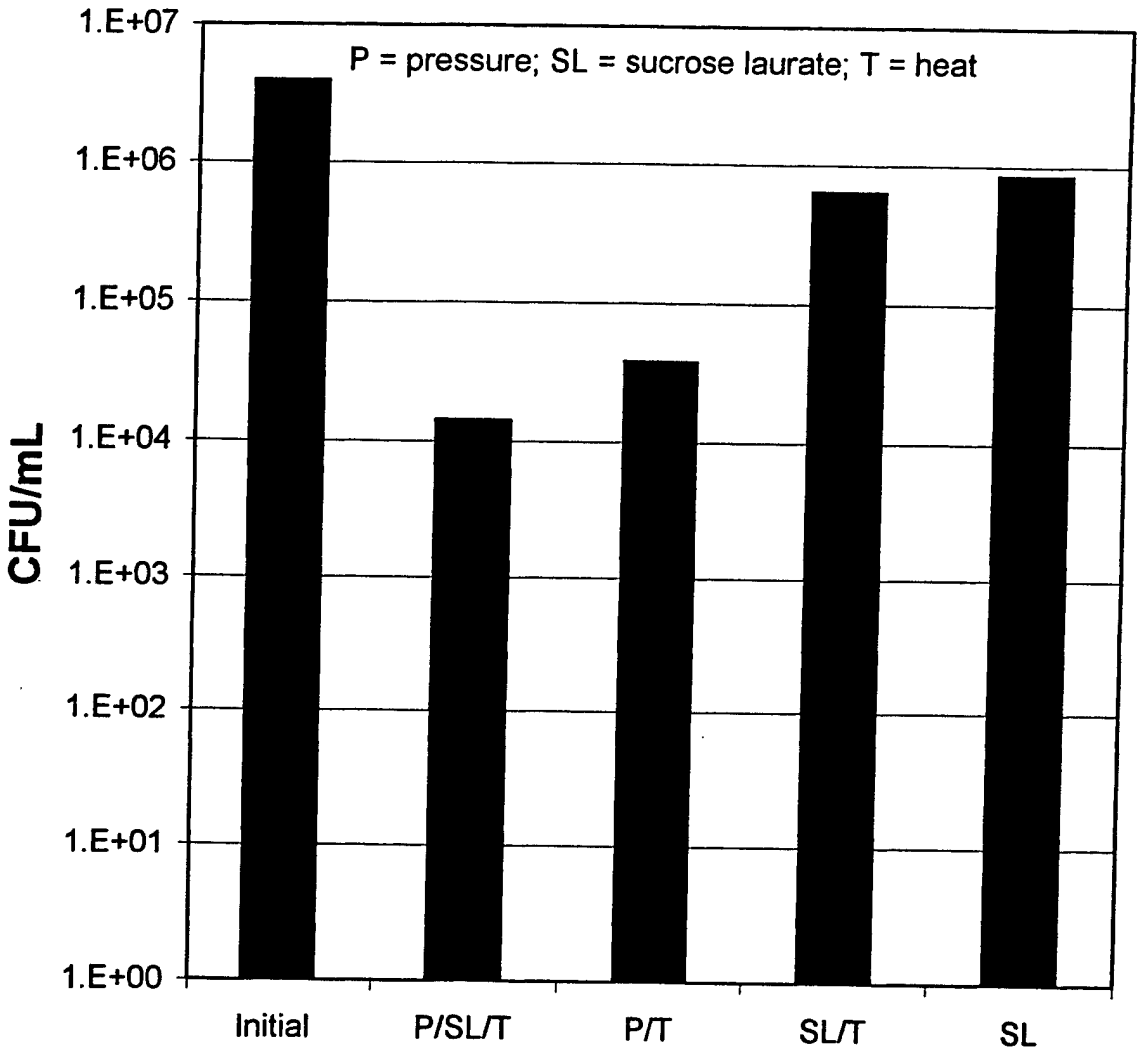

Fig. 10. Inhibition of *Alicyclobacillus* N1089 in apple juice by individual and combined treatments of pressure (392 MPa) for 10 min at 45 °C and sucrose laurate [0.04%(w/v) L1695], and plated on nutrient agar.

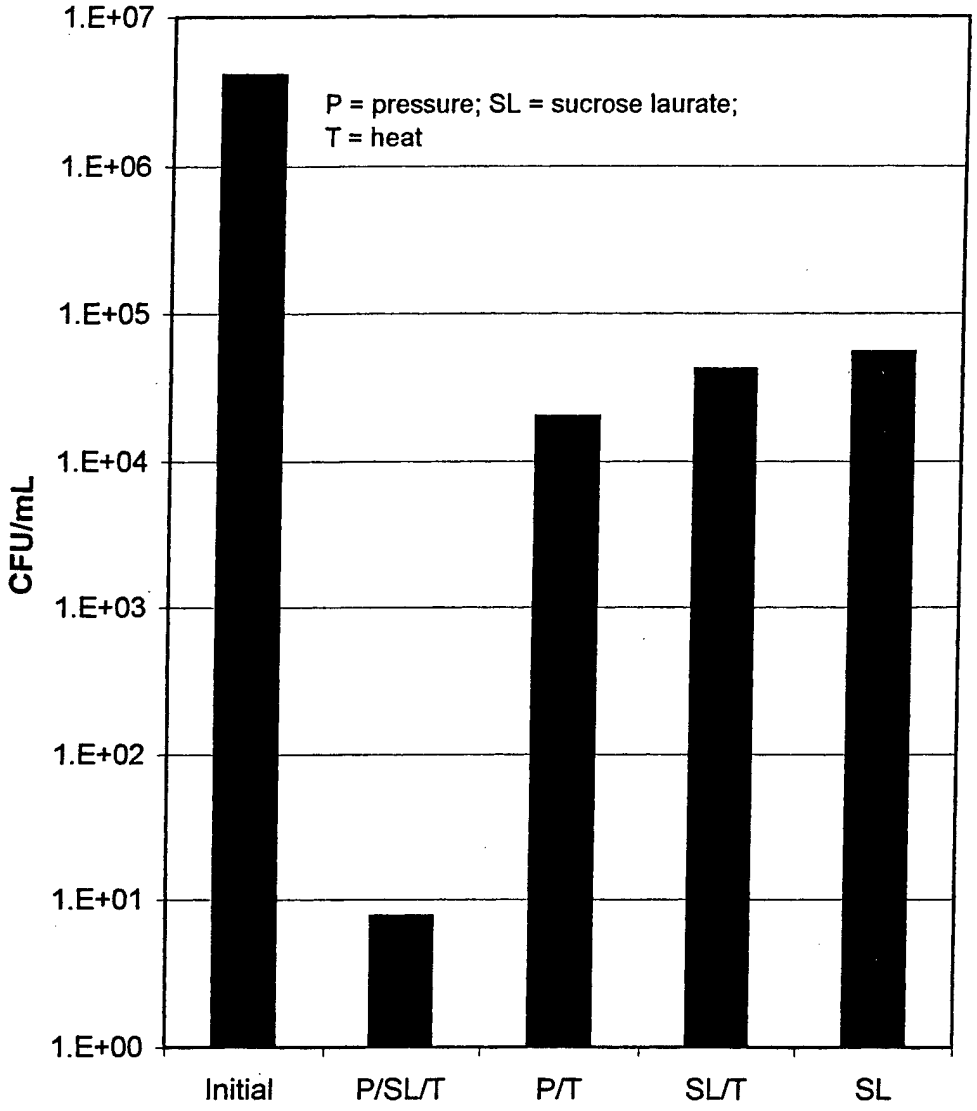

Fig. 11. Inhibition of *Alicyclobacillus* N1089 in tomato juice by individual and combined treatments of pressure (392 MPa) for 10 min at 45 °C and sucrose laurate [0.045% (w/v) L1695], and plated on nutrient agar.

CONCLUSIONS

MICs for sucrose laurate LWA1570 and Lauricidin™ without HPP or heat were determined for all cultures tested. The MIC of L1695 for most bacilli was >1% while *Alicyclobacillus* sp. and *C. sporogenes* PA3679 were inhibited by 0.5% L1695 or less. *B. stearothermophilus* 10149 generally demonstrated greater susceptibility to the sucrose esters than the other bacilli evaluated. The MICs of sucrose esters were higher than Lauricidin™. Sucrose stearates had little to no inhibitory effect on spore outgrowth except of *C. sporogenes* PA3679 and were soluble only with maintenance of elevated temperatures.

Treatment at 392 MPa for 10 to 15 min at 45 °C resulted in a 4.5- to 5.5-$\log_{10}$ reduction of 10^6 CFU/mL *B. subtilis* 168 in milk, *B. cereus* 14579 in beef, *B. coagulans* 7050 in tomato juice (pH 4.5), *Alicyclobacillus* N1089 in tomato juice (pH 4.5), and *Alicyclobacillus* N1098 in apple juice. Sucrose laurate L1695 had no obvious effect on the appearance or odor of the food products at the concentrations evaluated (up to 1%), but did cause foaming during mixing. The combination of all three processing treatments of sucrose laurate, HPP, and mild heat appeared necessary to inactivate and inhibit spores of *Bacillus* in this study. The concentration of L1695 needed to partially inhibit *B. coagulans* in tomato juice (pH 4.5) was approximately 100 times greater than the level needed to inhibit *Alicyclobacillus* N1089 in the same product. Complete inhibition of *Alicyclobacillus* N1098 was not achieved in apple juice at 0.045%; the MIC in KA without HPP or heat was just slightly higher at 0.05%. It is reasonable to expect that a slightly higher concentration of L1695 than 0.045%

would be adequate to inhibit *Alicyclobacillus* without added pressure or heat, but this must be verified experimentally.

Sucrose laurate appeared to inhibit spore outgrowth rather inactivate spores as indicated by the need for continual presence of sucrose ester after pressurization. The MIC of LWA1570 was lower than that of L1695, but the effectiveness in foods was ambiguous due to erratic plate counts and was possibly due to the dilution of milk during plating.

The inhibitory effects observed on sporeformers by the combined treatment of HPP, mild heat, and sucrose laurate appeared promising for food applications where alternatives to high heat processing are desired. The results warrant further investigation and adjustment of the parameters to attempt to achieve complete spore inhibition in specific food products.

This document reports research undertaken at the U.S. Army Soldier and Biological Chemical Command, Soldier Systems Center, Natick, MA, and has been assigned No. NATICK/TR-03/018 in a series of reports approved for publication.

REFERENCES

1. Baker, R.C., Poon, W., Kline, D. and D.V. Vadehra. 1982. Antimicrobial properties of Lauricidin in mechanically deboned chicken, minced fish and chicken sausage. *J. Food Safety* 4: 177-184.

2. Blaszyk, M. and R. A. Holley. 1998. Interaction of monolaurin, eugenol and sodium citrate on growth of common meat spoilage and pathogenic organisms. *Int. J. Food Microbiol.* 39: 175-183.

3. FDA (U.S. Food and Drug Administration). 1998. Bacteriological Analytical Manual, 8th edition. AOAC International, Gaithersburg, MD. Appendix 3.

4. Haas, G.J., Prescott, Jr., H. E., Dudley, E., Dik, R., Hintlian, C. and L. Keane. 1989. Inactivation of microorganisms by carbon dioxide under pressure. *J. Food Safety* 9: 253-265.

5. Hayakawa, I., Kanno, T., Tomita, M. and Y. Fujio. 1994a. Application of high pressure for spore inactivation and protein denaturation. *J. Food Sci.* 59(1): 159-163.

6. Hayakawa, I., Kanno, T., Yoshiyama, K. and Y. Fujio. 1994b. Oscillatory compared with continuous high pressure sterilization on *Bacillus stearothermophilus* spores. *J. Food Sci.* 59(1): 164-167.

7. Harwood, C. R. and S. M. Cutting (eds.). 1990. Appendix I-media. In: *Molecular Biological Methods for* Bacillus, p. 549, John Wiley and Sons, Chichester, England.

8. Hierholzer, J.C. and J.J. Kabara. 1982. In vitro effects of monolaurin compounds on enveloped RNA and DNA viruses. *J. Food Safety* 4: 1-12.

9. Hite, B.H. 1899. The effect of pressure in the preservation of milk. *W. Va. Univ. Agr. Exp. Stn. Bull. (Morgantown)* 58:15-35.

10. Kabara, J.J. 1993. Medium-chain fatty acids and esters. In: *Antimicrobials in Foods* 2nd edition. (P.M. Davidson and A.L. Branen, eds.). Ch. 8. pp. 307-342.

11. Kato, N. 1981. Antimicrobial activity of fatty acids and their esters against a film-forming yeast in soy sauce. *J. Food Safety* 3: 121-126.

12. Kimsey, H.R., Adams, D.M., and J.J. Kabara. 1981. Increased inactivation of bacterial spores at high temperatures in the presence of monoglycerides. *J. Food Safety* 3: 69-82.

13. Lisker, N. and N. Paster. 1982. Antifungal activity of monolaurin and related compounds. *J. Food Safety* 4: 27-34.

14. Monk, J.D., Beuchat, L.R. and A.K. Hathcox. 1996. Inhibitory effects of sucrose monolaurate, alone and in combination with organic acids, on *Listeria monocytogenes* and *Staphylococcus aureus*. *J. Appl. Bacteriol.* 81: 7-18.

15. Nakayama, A., Yano, Y., Kobayashi, S., Ishikawa, M. and K. Sakai. 1996. Comparison of pressure resistances of spores of six *Bacillus* strains with their heat resistances. *Appl. Environ. Microbiol.* 62(10): 3897-3900.

16. Nicholson, W. L. and P. Setlow. 1990. Sporulation, germination, and outgrowth. In: *Molecular Biological Methods for* Bacillus, C. R. Harwood and S. M. Cutting (eds.), pp. 391-450, John Wiley and Sons, Chichester, England.

17. Oh, D.H. 1993. Antimicrobial properties of glycerol monolaurate either alone or combined with selected organic acids against *Listeria monocytogenes*. Ph.D. Dissertation. The Louisiana State University, Baton Rouge, LA.

18. Oh, D.H. and D.L. Marshall. 1992. Effect of pH on the minimum inhibitory concentration of monolaurin against *Listeria monocytogenes*. *J. Food Prot.* 55(6): 449-450.

19. Oh, D.H. and D.L. Marshall. 1995. Destruction of *Listeria monocytogenes* biofilms on stainless steel using monolaurin and heat. *J. Food Prot.* 57(3): 251-255.

20. Okazaki, T., Kakugawa, K., Yamauchi, S., Yoneda, T. and K. Suzuki. 1996. Combined effects of temperature and pressure on inactivation of heat-resistant bacteria. In: *High Pressure Bioscience and Biotechnology*, R. Hayashi and C. Balny (eds.), pp. 415-417,Elsevier, Amsterdam.

21. Patterson, M.F., Quinn, M., Simpson, R. and A. Gilmour. 1995. Sensitivity of vegetative pathogens to high hydrostatic pressure treatment in phosphate-buffered saline and foods. *J Food Prot.* 58(5): 524-529.

22. Roberts, C.M. and D.G. Hoover. 1996. Sensitivity of *Bacillus coagulans* spores to combinations of high hydrostatic pressure, heat, acidity and nisin. *J. Appl. Bacteriol.* 81:363-368.

23. Shibasaki, I. 1982. Food preservation with nontraditional antimicrobial agents. *J. Food Safety* 4: 35-58.

24. Tsuchido, T., Saeki, T. and I. Shibasaki. 1981. Death kinetics of *Escherichia coli* in a combined treatment with heat and monolaurin. *J. Food Safety* 3: 57-68.

25. Wang, L.L. and E.A. Johnson, 1992. Inhibition of *Listeria monocytogenes* by fatty acids and monoglycerides. *Appl. Environ. Microbiol.* 58(2):624-629.